Detection of New Delhi Metallo-β-lactamase-1 (NDM-1) Gene from Multidrug-resistant *Pseudomonas aeruginosa* Isolated from Dhaka Medical College Hospital

A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY IN PARTIAL FULFILMENTOF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

This is to declare that the research work embodying the results reported in this thesis entitled "Detection of New Delhi Metallo-β-lactamase-1 (NDM-1) Gene from Multidrug-resistant *Pseudomonas aeruginosa* Isolated from Dhaka Medical College Hospital" submitted by Md. Ruhul Amin has been carried out under the supervision and guidance of Dr. M. Mahboob Hossain, Professor, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University in partial fulfilment of MS. in Biotechnology, at BRAC University, Dhaka. It is further declared that the research work presented here is original, has not been submitted anywhere else for any degree or diploma.

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DEDICATION

To

My Beloved Parents

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Md. Ruhul Amin

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CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

> =More than

>= Equal or more than

<= Equal or less than

59be= 59-base element

ATCC= American type culture collection,

BIRDEM= Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine, and Metabolic Disorders.

bla= Beta-lactamase gene

BSMMU= Bangabandhu Sheikh Mujib Medical University

CDC= Centre for Disease Control and Prevention

CFU= Colony forming unit

CLSI= Clinical and laboratory Standard Institute

CTX-M= Active on cefotaxime

DDS= Double-disk synergy

DMCH= Dhaka Medical College Hospital

DNA= Deoxyribonucleic acid

dNTP= Deoxynucleotide Triphosphate.

E test= Epsilometer

EDTA= Ethylene diamine tetra acetia acid

ERC= Ethical review committee

ESBL= Extended spectrum β-lactamase

Et al. =et alia (and others)

icddr, b= International Centre for Diarrhoeal Disease Research, Bangladesh

ICU= Intensive care unit

IMP=Imipenemase

ISC1= Insertion sequence common region

KIA= Kligler iron agar

LCR= Ligase chain reaction

MBL= Metallo-β-lactamase

MDR= Multidrug resistant

MgCl2= Magnesium chloride

MHT= Modified Hodge test

MIC= Minimum inhibitory concentration

MIU= Motility -indole -urea

MPA= Mercaptopropionie acid

NCCLS= National Committee for Clinical Laboratory Standard

NDM-1= New Delhi metallo-β-lactamase-1

NMC= Non metallo carbapenemase

OXA= Oxacillinase

PBP= Penicillin-binding-protein

pCMB= p-chloromercuribenzolate

PCR= Polymerase chain reaction

PFGE= Pulsed field gel electrophoresis

PSE= Pseudomonas- specific enzyme

RFLP= Restriction fragment length polymorphism

RRC= Research review committee

SHV=Sulfhydryal variabale

SPM= Sau Poulo MBL

TBE= Tris-Borate-EDTA

TE= Tris-EDTA

TEM= Temoniera

UTI= Urinary tract infection UV= Ultra violate

V/v= Volume/volume

VIM= Verona integron-encoded metallo-β-lactamase

W/v= Weight/volume

Zn= Zinc

ABSTRACT

New Delhi Metallo-beta-lactamase-1 (NDM-1) producing superbugs create a global public health problem because of their resistance to most of the antibiotics. This study was conducted to determine the presence of NDM-1 producers in carbapenem-resistant *Pseudomonas aeruginosa* isolated from Dhaka Medical College Hospital, Bangladesh. Out of 120 gram-negative bacteria isolated from wound swab, 58 (48.33%) *were Pseudomonas aeruginosa,* 28 of which were from burn unit and 30 were from medicine, surgery, gynecology and obstetrics units. *Pseudomonas aeruginosa* isolated from burn unit showed more resistance to all other antibiotics than organism isolated from other units. Extended spectrum-β-lactamase (ESBL) producers were detected by double disk synergy test (DDS) and Metallo-β-lactamase (MBL) producers were detected by both Hodge test and double disk synergy test. Total 38 (31.67%) isolates were detected as ESBL producers and 40 (33.33%) were detected as MBL producers. In the case of *Pseudomonas aeruginosa* 17 (29.31%) were ESBL producers and 20 (34.48%) were MBL producers.

Total 24 (41.37%) carbapenem-resistant *Pseudomonas aeruginosa* were detected by the disk diffusion test of which 15 were from burn unit and 9 from other units. Carbapenem-resistant *Pseudomonas aeruginosa* were screened for the presence of NDM-1 by PCR and 6 (25%) were found positive for *bla*NDM-1. In the case of the burn unit, 33.33% of carbapenem-resistant *Pseudomonas aeruginosa* were NDM-1 producer. NDM-1 producing *Pseudomonas aeruginosa* were found 100% resistant to all commonly used antibiotics except colistin.

The result of this study provided insights into the high proportion of NDM-1 producers among *Pseudomonas aeruginosa* in Dhaka Medical College Hospital and the importance of rational use of antibiotics.

1. INTRODUCTION

Members of the bacterial genus Pseudomonas, especially *Pseudomonas aeruginosa (P. aeruginosa*) are among the major nosocomial pathogens because of their ubiquitous nature and ability to colonize and survive in hospital reservoirs (Alous *et al.,* 2006). This organism is clinically important since it possesses several virulence factors and is intrinsically resistant to most of the antimicrobial and disinfectant agents (Gales *et al.,* 2003).

P. aeruginosa infections are associated with increased mortality and morbidity, especially in immunocompromised and burn patients (Mikulska *et al.,* 2009; Navon-Venezia *et al.,* 2005). *P. aeruginosa* represents a phenomenon of bacterial resistance, and demonstrate particularly all known enzymatic and mutational mechanisms of bacterial resistance (Pechere and Kohler, 1999). These characteristics are caused by the selective pressure of mutations in chromosomal genes that lead to ampC hyperexpression, repression or inactivation of oprD, and overexpression of efflux pumps (Livermore, 2002). In addition, *P. aeruginosa* is able to acquire another drug-resistance determinant by horizontal transfer of mobile genetic elements coding for class B carbapenemases, also called Metallo-β-lactamases or MBLs, which hydrolyze all β-lactams except aztreonam (Queenan and Bush, 2007).

Molecular classification of β-lactamases is based on the nucleotide and amino acid sequence in these enzymes (Ambler, 1980). To date, four classes are recognized (A-D), correlating with the functional classification defined by enzyme substrate and inhibitor profile (Bush *et al.,* 1995).

Carbapenems are a group of broad-spectrum antibiotics which are not inactivated by commonly encountered β-lactamases including AmpC and very effective against a

wide range of bacteria (Livermore and Yang, 1989). Often they are used as last resort against multi-drug resistant (MDR) bacteria particularly in a critical care setting.

Resistance to carbapenem is due to decreased outer membrane permeability, increased efflux system, alterations of penicillin-binding proteins and carbapenemases (Gladstone *et al.,* 2005). These carbapenemases are class B Metallo β-lactamases(IMP, VIM) or class D-oxacillinases (OXA 23 to OXA 27) or class A - clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC) (Gladston *et al.,* 2005).

MBLs belong to molecular class B that correspond group 3 (Ambler, 1980; Bush et al, 1995) and they are capable to hydrolyze all classes of β-lactams except monobactams and not inhibited by β-lactamase inhibitors such as clavulanic acid (Bush *et al.,* 2005; Walsh *et al.,* 2005). As they require zinc (Zn^{2+}) for degradation of β- lactam antibiotics, metal chelators such as ethylene diamine tetraacetic acid (EDTA), 0-1, 10-phenanthroline, dipicolinic acid and mercaptopropionic acid (MPA) are able to inhibit these metallo-enzymes (Toney and Moloughney, 2004). The genes responsible for MBL production may be chromosomally or plasmid-mediated and hence pose a threat of the spread of resistance by gene transfer among the gram-negative bacteria (Gladston *et al.,* 2005).

Plasmid-borne MBLs are IMP-1 to 30, VIM 1to 30, SPM-1, GIM-1, SIM-1, and the new variant, New Delhi Metallo-β-lactamase-1 (NDM-1) (Bebrone, 2007; Young *et al.,* 2009; Jacoby and Bush, 2011). MBL genes are normally encoded in class 1integrons along with other resistant determinants, such as the aminoglycoside-modifying enzymes. The integrons are frequently located in plasmids or transposons, the dissemination of which contributes to the global spread of this resistance mechanism (Poirel *et al.,* 2000, Riccio *et al.,* 2001)

Chromosomally encoded MBLs are primarily found in environment isolates of *Aeromonas spp, Strenotrophomonas maltophilia, Bacillus spp*, etc. which are of low pathogenic potential (Walsh, 2005). The VIM type carbapenemase resistant has been found most commonly in strains of less commonly encountered bacteria such as *P. aeruginosa, Acinetobacter baumannii, Aeromonas hydrophila* and *Enterobacteriaceae* family (Cornagila *et al.,* 2011; Walsh *et al.,* 2005).

VIMs and IMPs are the most frequent MBLs acquired by gram-negative bacilli (Zhao and Hu, 2010). VIM-1 (Verona integron encoded Metallo-β-lactamase) carbapenemase found in *P. aeruginosa* strain isolated at the Verona University Hospital, Italy, in1997, is the first representative of a new family of acquired MBLs (Laurette *et al.,* 1999). VIM-2 was originally identified in a *P. aeruginosa* bloodstream isolates from a patient with neutropenia in Marseille (South France) (Poirel *et al.,* 2000). Since its first report from France, VIM-2 has been reported worldwide and become the most prevalent Metallo-β-lactamase (Walsh *et al.,* 2005).

NDM-1 represent the recent type of mobile Metallo-β-lactamase carbapenemase to appear but it is behaving differently than most of the other previously described mobile serine or Metallo-β-lactamase carbapenemase, in terms of rapidity of its spread and the scope of the organism in which it is found (Kumarasamy *et al.,* 2010). New Delhi-Metallo-β-lactamase (NDM-1) producer are also referred to as ‘superbug' because of treatment failure by broad-spectrum antibiotics (Salcido, 2010).

The first published report and description of NDM-1 were in 2009, a Swedish patient receiving treatment for a *K. pneumoniae* urinary tract infection in a Swedish hospital, who had previously been hospitalized in New Delhi, India. The gene was found on an easily transferable plasmid within the *K. pneumoniae* strain, it was also found on a plasmid in an *E. coli* strain isolated from the patient’s feces (Yong *et al*.,

2009). In addition to the presence of NDM-1 in multiple members of the *Enterobacteriaceae* family, it has now been reported in multiple other gram-negative bacteria including *Acinetobacter baumannii, Vibrio cholera, Shigella boydii, Aeromonas caviae*, and *p. aeruginosa* (Espinal *et al*., 2011; Cheny *et al*., 2011; Walsh *et al*., 2011).

NDM-1 carrying isolates are resistant to carbapenems, β-lactam antibiotics, aminoglycosides and fluoroquinolone which have been the mainstays for therapy of enteric gram-negative bacilli infections (Kumarasamy *et al*., 2010). NDM-1 gene was most frequently associated with plasmids and was sometimes carried on more than one plasmid (Poirel *et al*., 2011). Some isolates had *bla*NDM-1 gene found on the chromosome. Collectively these findings suggested considerable mobility of the gene (Kumarasamy *et al*., 2010).

The ability of NDM-1 to spread not only among Enterobacteriaceae but also among other bacterial families, like *Pseudomonaceae*, implies the possibility for numerous new NDM-1 cases to be detected in the near future. MBL producing gram-negative bacilli, especially *Pseudomonas spp.* have been increasingly reported in Asia, Europe, Latin America and the United States (Chu *et al.,* 2001; Toleman *et al.,* 2004). Therefore, detection of MBL - producing gram-negative bacilli is crucial for the optimum treatment of patients and to control the spread of resistance.

The prevalence of imipenem resistant MBL producers is 43% to 83.1% for Pseudomonas species in Bangladesh (Anwar *et al.,* 2010; Nasrin *et al.,* 2010). Previously 87.50% imipenem resistant Pseudomonas species isolated at Dhaka Medical College Hospital (DMCH) are found to be MBL producers (Farzana *et al.,* 2013). About 3.5% of imipenem resistant gram-negative bacteria isolated at icddr,b was NDM-1 producer in Bangladesh (Islam *et al.,* 2011) and 22.86% NDM-1

producer was reported among gram-negative bacteria isolated at DMCH (Farzana *et al.,* 2013)

Several molecular methods have been described for detection of MBLs producers. PCR is considered as the most sensitive method for detection of MBLs producers by amplifying specific genes responsible for MBLs production by the bacteria. However, this technique cannot distinguish the type of variants which can be confirmed by sequencing (Walsh *et al.,* 2005).

A very little is known about the molecular detection of MBLs in Bangladesh. MBL producing Pseudomonas is reported by EDTA-imipenem (EDTA-IMP) agar dilution MIC reduction method (Nasrin *et al*., 2010). This study is designed for molecular detection of MBL encoding gene *bla*NDM-1 among multidrug-resistant *P. aeruginosa* by PCR.

Research Hypotheses

*bla*NDM-1 genes are responsible for multidrug resistance.

Objectives

General Objective

To detect MBL encoding gene *bla*NDM-1 among multidrug resistant *Pseudomonas aeruginosa.*

Specific Objectives

To isolate and identify gram-negative bacteria by culture and biochemical tests collected from Dhaka Medical College Hospital (DMCH).

To determine antimicrobial susceptibility patterns of isolated gram-negative bacteria.

To detect the *bla*NDM-1 gene by PCR among the multidrug resistant *Pseudomonas aeruginosa.*

2. REVIEW OF LITERATURE

The gram-negative bacteria are showing slow but steady upward trends of resistance as compared to gram-positive bacteria and this has prompted the health authorities to curb their dissemination. The main problem with these gram-negative bacterial infections is their effective treatment. The β-lactam group of antibiotics that includes penicillins, cephalosporins, monobactam, and carbapenems form the mainstay of therapy. The production of β-lactamase by gram-negative bacteria is the major defense mechanism against these β-lactam antibiotics (Medeiros, 1997). The bacteria have responded by producing a plethora of newer β-lactamases like extended spectrum β-lactamases (ESBLs), plasmid-mediated Amp-C enzymes, and carbapenem hydrolyzing beta-lactamases (Gniadkowski, 2001).

The overall prevalence of these Metallo β-lactamases among clinical isolates has remained low since most Metallo β-lactamases are encoded by genes resident in species that are of minor clinical relevance and that are not transferable to major bacterial pathogens (Bush, 1998; Livermore, 1995; Rasmussen and Bush, 1997). Recently, however, the spread of acquired Metallo β-lactamase gene like *bla*IMP, *bla*VIM has been reported among isolates from various members of family *Enterobacteriaceae, P. aeruginosa,* and other nonfastidious gram-negative nonfermenters (Ito *et al.,* 1995; Senda *et al.,* 1996).

P. aeruginosa is a gram-negative rod unable to ferment glucose and is widely known as an opportunistic organism, frequently involved in infections of immunosuppressed patients, and also causes outbreaks of hospital-acquired infections (Sader *et al*., 2001).

This organism is clinically important since it possesses several virulence factors and is intrinsically resistant to most of the antimicrobial and disinfectant agents, a feature that is also responsible for difficulties in treating infected patients (Gales *et al.,* 2003). The increased involvement of this ubiquitous organism in infections is due to a number of factors, including the growing numbers of invasive procedure and immunocompromised patient together with the increased use of antibiotics, which has promoted the selection of resistant organisms. Patients in intensive care units, oncology departments, burn units, and surgery wards frequently show multidrug-resistant isolates, which contribute to high morbidity and mortality (Giamarellos *et al.,* 2006).

Intrinsic resistance properties of *Pseudomonas spp.* reflect the synergy between the bacterium's low outer-membrane permeability (Angus *et al.,* 1982; Dorner *et al.,* 2009; Yoshimura and Nikaido, 1982), its chromosomally encoded AmpC β-lactamase (Livermore, 1987), and its broadly specific drug efflux pump (Kohler *et al.,* 1997; Masuda *et al.,* 2000; Poole *et al.,* 1996; Poole *et al*., 1993). Furthermore, *P. aeruginosa* readily acquires resistance to most antimicrobials through mutations in its chromosomal genes and through extrachromosomal elements carrying resistance determinants (Livermore and Yang, 1987).

A major structural component of a bacterial cell wall, the peptidoglycan layer contains a chain of 10 to 65 disaccharide residues composed of alternating N-acetylglucosamine and N-acetylmuramic acid which are cross-linked with Peptide Bridge. At the time of exposure of growing bacteria to β-lactam antibiotics, drugs bind with specific penicillin-binding proteins (PBPs) and halt cross-links of peptidoglycan chains. Cells become eventually dead by activating the autolysis (murein hydrolases) in drug-treated cells. Bacteria may confer resistance to this group

of drugs by means of preventing interaction to target PBPs or modifying PBPs or generating β-lactamases (Murray *et al.,* 2009). Production of β- lactamases is the major cause of bacterial resistance to β-lactam antibiotics (Stratton, 2000).

The carbapenems

Carbapenems are a class of β-lactam antibiotic with a broad spectrum of antibacterial activity. They have a structure that renders them highly resistant to most of the β-lactamases including the extended spectrum β-lactamases and AmpC β-lactamases. Carbapenem antibiotics were originally developed from thienamycin, a naturally derived product of *Streptomyces cattleya.* The carbapenems traverse the outer membrane barrier of bacteria by OprD protein and bind the penicillin-binding proteins (PBPs) to produce their effect on a wide range of gram positive and gram negative bacteria and are incredibly stable to many β-lactamases. Commonly used antibiotics in this group are imipenem, meropenem, ertapenem etc.

Carbapenems are generally used as a last resort to treat serious infections caused by *P. aeruginosa*, but the emergence and spread of acquired carbapenem resistance in this species have challenged the success of therapeutic and control efforts. However, carbapenem resistance has been observed frequently in non-fermenting bacilli like *P. aeruginosa* and *Acinetobacter spp.*

Carbapenem resistance

Carbapenem resistance is partly mediated by loss of OprD protein (initially called D2 porin). The primary role of this protein is the passive uptake of basic amino acids across the outer membrane, but it forms pores that are also permeable to carbapenems though not to other β-lactams (Huang and Hancock, 1993). The multidrug efflux systems which mediate the resistance to quinolone, chloramphenicol and many other antimicrobial agents, also contribute the carbapenem resistance. The strains which

overexpress the MexAB-OprM system or express the MexEF-OprN system exhibit the carbapenem resistance by pumping the drag out. In addition to the OprD loss or drug efflux pumps, chromosomal AmpC β-lactamase plays an important role in carbapenem resistance in *P. aeruginosa.* Loss of OprD determines resistance to carbapenems only in cases of expressed chromosomal AmpC β-lactamase, and this demonstrates the close co-operation between these two mechanisms (Livermore, 1992).

Loss of OprD is associated with resistance to imipenem and reduced susceptibility to meropenem. To complicate matters, OprD is co-regulated with MexEF-OprN; thus *nfxc (mexT)* mutants that occasionally are selected by fluoroquinolones (not carbapenem) have (1) up-regulated MexEF-OprN and reduced OprD with consequent resistance to both fluoroquinolones and imipenem, and (2) reduced susceptibility to meropenem (*Ochs et al.,* 1999). Imipenem does not appear to be a substrate for MexAB-OprM, but because of its hydrophobic side chain, meropenem can be affected by this system (*Kohler et al.,* 1999; *Li et al.,* 1994; *Pai et al.,* 2001). The interaction of meropenem with these resistance mechanisms differ from those of imipenem. It is inferred that meropenem, like imipenem, can use OprD pathway to enter the *Pseudomonal* cell but, unlike imipenem, it is recognized and ejected by MexB-mediated efflux, presumably because of its 2′ heterocyclic side chain, likely to cause resistance in *P. aeruginosa*.

These differences between imipenem and meropenem allow arguments for either compound as ‘the less likely to cause resistance in *P. aeruginosa*. The case for imipenem argues that it selects OprD mutants; these have a narrow spectrum insensitivity and remain fully susceptible to other drugs, whereas meropenem resistance co-depends on upregulation of MexA-MexB-OprM, a mechanism that

compromises fluoroquinolones as well as other β-lactams (Livermore, 1992; Arita *et al.,* 1999). The case for meropenem is that substantive resistance is much harder to achieve than to imipenem since two mutations (loss of OprD and upregulation of MexA-MexB-OprM) are needed rather than one. Upregulation of another efflux system, MexE-MexF- OprN is associated with raised MIC of both carbapenems as well as fluoroquinolones (Maseda *et al.,* 2000). Finally, Metallo-β-lactamases of IMP and VIM families are increasing source of resistance to carbapenems in *P. aeruginosa* (Livermore and Wodford, 2000).

In the 1980s, the advent of the usage of a new β-lactam group of antibiotics i.e., carbapenems, heralded a new therapeutic option for the serious gram-negative infections, but soon emerged the enzymes, which could destroy even these carbapenems and pan drag resistant strains began to emerge (Hsueh *et al.,* 2002). These carbapenem hydrolyzing β-lactamases i.e. carbapenemases are the diverse group of enzymes. They are mainly of two types - first types are the serine carbapenemase belonging to molecular class A or D of Ambler. Class A clavulanic acid inhibitory enzymes (SME, NMC, IMI), plasmid-mediated KPC enzymes and class D oxacillinases (OXA 23 to OXA 27) type β-lactamases which exhibit carbapenemase activity are included in this group only. The second type the Metallo β- lactamases (MBL) belong to Ambler Class B and they differ from other carbapenemases in having broad substrate profile, the potential for horizontal transfer and lack of inhibition by serine β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Walsh *et al.,* 2000).

Ambler first separately classified MBL's from serine β-lactamases in 1980 (Ambler, 1980). Bush further classified them into a separate group (Group 3; Table 1) based on their functional properties like substrate profiles, sensitivity to EDTA and lack of

inhibition by serine β-lactamase inhibitors (Bush, 1989). This scheme was updated in 1995 to accommodate the growing number of group 3 enzymes (Bush *et al.,* 1995). Rasmussen and Bush proposed three functional subgroups of MBL's on the basis of imipenem and others β-lactam hydrolysis (Rasmussen and Bush, 1997). Group 3a enzymes are those that hydrolyze penicillin as fast as or faster than they hydrolyze imipenem. They have a broad spectrum of activity. Cephalosporins are also hydrolyzed by these enzymes but not as well as imipenem. The subgroup 3b is the enzymes that have high specificity for hydrolyzing carbapenems. The third subgroup 3c has high cephalosporinase activity.

According to molecular classification, MBLs are a disparate group of enzymes. Based on the combination of structural features, zinc affinities for the two binding sites and hydrolysis characteristics the MBLs are currently classified into three different lineages, identified as subclasses Bl, B2 and B3.

MBLs, like all β-lactamases, can be divided into those that are normally chromosomally mediated and those that are encoded by transferable genes. Some bacteria, usually from environmental habitats, ubiquitously carry MBLs, although there is much debate as to why this is the case. One argument is that over a considerable period of time, the bacteria have exposed to β-lactam or β-lactam type compounds and the bacteria have been conscripted to acquire and maintain these genes and their products. Another argument is that these enzymes perform a natural cellular function that is yet to be fully elucidated. Regardless of the viewpoint, a number of these MBL genes are inducible and the majorities of the bacteria carrying them are or can become highly resistant to β-lactams. Fortunately, these organisms are opportunistic pathogens and with an arguable exception of *S. maltophilia and Bacillus anthracis,* seldom cause serious infections. Chromosomally encoding MBL bacteria

include *B. cereus* (BC II), *Bacillus anthracis, S. maltophilia* (L1), *Aeromonas hydrophilia* (CphA), *Chryseobacterium meningosepticum* (BlaB or GOB-1), *Chryseobacterium indologenes* (IND-1), *Legionella gormanni* (FEZ-1), *Caulobacter crescentus* (MbIIB), *Myroides spp.* (Tus-1, Mus-1) etc. Generally speaking, the chromosomal MBLs from a particular species or genus vary little from one another and are often co-regulated with serine β-lactamases. Based on amino acid sequence homology, types of acquired MBLs include the IMP and VIM, SPM-1, GIM-1, SIM-1, AIM-1, KHM-1, NDM-1 and SID-1 (Cornaglia *et al.,* 2011; Walsh *et al.,* 2005).

MBLs and serine β-lactamase both mediate resistance to β-lactams by cleaving the amide bond of the β-lactam ring; however, the way in which the two groups of enzymes achieve this differ considerably (Frere, 1995). MBLs possess a distinct set of amino acid that defines the finite architecture of the active site which coordinates the zinc ions. The zinc ions in turn usually coordinate two water molecules necessary for hydrolysis (Wang *et al.,* 1996). The principal zinc-binding motif is histidine-X-histidine-X-aspartic acid, which is common to most MBLs apart from the class B2 enzymes (Rasmussen and Bush, 1997). Most MBLs accommodate two zinc ions in their active sites; the class B2 enzymes possess just a single zinc ion (Rasmussen and Bush, 1997). The proposed mechanism for hydrolysis suggests that the active site orients and polarizes the β-lactam bond to facilitate nucleophilic attack by zinc-bound water/hydroxides (MacManus and Crowdwe, 1999; Spencer *et al.,* 2001; Wang and Benkovic, 1998). Chelators of zn^{2+} such as EDTA, 0-1, 10-phenanthroline and dipicolinic acid are able to inhibit these metallo-enzymes (Toney and Moloughney, 2004).

The most common and widespread acquired MBLs are those of IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are

known. VIMs are composed of 30 members of metallo-enzymes (Jacoby and Bush, 2011). The first identified VIM enzyme, VIM -2 was discovered in 1996 from Marseille (South France) from a *P. aeruginosa* bloodstream isolate from a patient with neutropenia (Poirel *et al.,* 2000). Subsequently, in 1997 VIM-1 was identified from Verona, Italy.

History of NDM-1

NDM-1 is a novel MBL found in *Enterobacteriaceae.* It was first reported in a Swedish patient of Indian origin, a diabetic male who returned to Sweden after receiving treatment at a hospital in New Delhi for a gluteal abscess in December 2007 (Yong *et al.,* 2009). In January 2008, the patient visited a Swedish hospital where a *Klebsiella pneumonia* carrying the novel MBL was isolated from his urine. Fecal cultures done to identify the reservoir of infection yielded a strain of *Escherichia coli* carrying a similar resistance factor suggesting the possibility of conjugational transfer in the gut. Since then, *Enterobacteriaceae* isolates harboring the NDM-1 gene have been found in multiple areas of India, Pakistan, Bangladesh and also in the USA, Canada, China, Japan and the United Kingdom (Sarma *et al.,* 2011). NDM-1 producers are now referred to as ‘superbug' in the news media because of resistance against broad-spectrum antibiotics. This new MBL containing organisms are usually sensitive to polymyxins and tigecycline (Kumarasamy *et al.,* 2010). Organisms bearing *bla*NDM-1 gene have been identified from Europe (Kumarasamy *et al.,* 2011; *Struelens et al.,* 2010), Australia (Poirel *et al.,* 2010), America (Mulvey *et al.,* 2011), Africa (Poirel *et al.,* 2011) and Asia (Kumarasamy *et al.,* 2010; Islam *et al.,* 2011).

NDM-1 share only 32.4% amino acid sequence homology to closely related VIM-1/VIM-2 MBL producers. Two aspects should raise concern for NDM-1 producing organisms. One is that this is difficult to treat resistance mechanism which was

observed in *Enterobacteriaceae*, the commonest normal intestinal flora. Two, this new NDM gene is carried on plasmids and on a cassette which means they can theoretically move as independent entities and be freely exchanged. These two reasons are sufficient to make this a major community-acquired problem if not sufficiently addressed. The plasmid carrying the *bla*NDM-l gene also carry a number of other genes conferring resistance to all aminoglycosides, macrolides, and sulfamethoxazole, thus making these isolates multidrug-resistant or because of other non-plasmid-mediated resistances, resistant in some cases to all antibiotics, including tigecycline and colistin (Kumarasamy *et al*., 2010).

Recently, Espinal *et al* (Espinal *et al*., 2011) identified a new variant of NDM-1 in *Acinetobacter baumannii* and designated as NDM-2. They reported that the clonal dissemination of an NDM-2 producing *A. baumannii* was isolated in an Israeli rehabilitation ward. Kasse *et al* (Kasse *et al*., 2011) revealed the sequence of NDM-2 and that variant had a C to G substitution at position 82 resulting in an amino acid substitution of proline to arginine.

In addition to the presence of NDM-1 in multiple members of the *Enterobacteriaceae* family, it is now been reported in multiple other gram-negative bacteria including *Acinetobacter baumannii, Vibrio cholerae, Shigella boydii, Aeromonas caviae and P. aeruginosa* (Espinal *et al.,* 2011; Chen *et al*., 2011; Jovcic *et al*., 2011; Walsh *et al.,* 2011). The presence of NDM-1 gene in 2 of 50 water sample of drinking water and 51 of 171 seepage samples from New Delhi including several new bacterial species in which NDM-l has not previously been reported was disconcerting from a public health perspective, especially given its presence in species of *Vibrio* and *Shigella,* providing an enormous potential for widespread dissemination (Walsh *et al.,* 2011). Collectively these later finding suggest a widespread presence of the plasmids

containing the NDM-1 gene in an environmental setting from an endemic area, a worldwide spread of the plasmids containing the NDM-1 gene, the beginnings of dissemination within non-endemic countries and a spectrum of presence in multiple species of gram-negative bacteria, some of which are major pathogens from a public health perspective.

NDM-1 producers bring several additional factors which are deeply disconcerting for public health worldwide. First, the *bla*NDM-l gene has been identified in not a single species but in unrelated species (including *Acinetobacter spp.)* (Karthikeyan *et al.,* 2010) indicating that this gene can spread at an unprecedented rate. Second, it is present not only in *K. pneumoniae*, a typical nosocomial pathogen but also in *E. coli,* which is a major community-acquired pathogen. Third, *E. coli* is also the number one cause of diarrhea in children in the subcontinent, increasing the risk of resistant strains being released into the environment. Overpopulation, lack of basic sanitation and access to clean water, a tropical climate, and poor control of antibiotic use are all compounding factors that may promote the transfer of NDM-1 positive bacteria among the populations. Therefore, there is an urgent need to detect NDM-1 producers in any health care facility to prevent their further spread.

The presence of NDM-1 on a very mobile plasmid, its presence on different plasmids, the ability to integrate into chromosome and co-carriage of so many resistance traits set it apart from other resistance traits we have encountered previously.

Transmission of Metallo-β-lactamases

Many studies characterizing of MBLs *bla* genes have found them inserted into common class I integron (Brizio *et al.,* 2006; Poirel *et al.,*2000; Pounaras *et al.,* 2002; Shibata *et al.,* 2003; Walsh *et al.,* 2003). These integrons are responsible for the transfer of *bla* genes among divergent species of gram-negative bacteria. Integrons are

capable of procuring gene cassettes via a site-specific recombination event between two DNA sites, one in the integron and one in the gene cassette. The integron consists of three regions: the 5′ conserved region, the 3′ conserved region, and a variable region. The 5′ region consists of the integrase gene (*intl*), its adjacent recombination site *(attl*) and a promoter, which facilitates expression of the procured gene cassette in the variable region. The 3′ conserved region often consists of a partially deleted *qac* gene (qacE 1) fused to a sul gene and correspondingly, confers resistance to antiseptics and sulfonamide, respectively.

Gene cassette is small pieces of circular DNA, approximately 1 kb in size, comprising a single gene together with a recombination site termed a 59-base element (Bennett, 1999). Cassette integration usually occurs at the attI site of integron and the original cassette 59 be is reformed whenever the gene cassette is excised from the integrons. Thus it is known that the resistance genes found on gene cassettes are potentially highly motile (Collis and Hall, 1995). Integrons are the genetic elements that are unable to move but they contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome. They spread from one organism to another with the help of plasmids or transposons and this dissemination contributes to the global spread of this resistance mechanism. Most of the genes encoding IMP, VIM as well as GIM are found as gene cassette in class 1 integrons. The gene which encodes NDM-1 is located on a transmissible plasmid and is associated with other resistant determinants.

Prevention of transmission of MBL producers

Antimicrobial resistance is a growing problem and is likely to become worse. Based on published literature, it appears that the resistance rate in gram-negative bacilli is higher in India and Asia as compared to the west (Hawkey, 2008; Gupta, 2007). A

majority of resistance is acquired under selection pressure. The increase in resistance of gram-negative bacteria is mainly due to mobile genes on plasmids that can readily spread through bacterial populations. Standardized plasmid typing methods are enhancing our understanding of the host ranges of these elements and their worldwide distribution (Carattoli, 2009; Carattoli *et al.,* 2006). Moreover, unprecedented human air travel and migration allow bacterial plasmids and clones to be transported rapidly between countries and continents (Walsh, 2006; Hawkey and Jones, 2009). Much of this dissemination is undetected, with resistant clones carried in the normal human flora and only becoming evident when they are the source of endogenous infections. The spread of these resistance genes is strongly facilitated by the following conditions:

- Low-level hygiene
- Overpopulation
- Hot, humid climate
- Widespread over-the-counter use of antibiotics.

The prevention of the spread of carbapenemase producers relies on early detection of carriers (Miriagou *et al.,* 2010; Nordmann *et al.,* 2011). Patients who undergo screening should include patients who were hospitalized while abroad and then transferred to another country and patients at risk (e.g., patients in intensive care units, transplant patients, immunocompromised patients). Screened patients should be kept in strict isolation before obtaining the results of the screening (at least 24-48 hours). Because the reservoir of carbapenemase producers remains in the intestinal flora, fecal and rectal swab specimens are adequate for performing this screening. Those specimens may be plated directly on screen media.

There is no universal screening medium able to detect all types of carbapenemase producers with high sensitivity and high specificity, however, agar plates containing imipenem at a concentration of 1 mg/L have been proposed for screening only KPC producers (Adler *et al.,* 2011). A culture medium designed to screen for ESBL producers (Chrome ID ESBL) may be used also for screening carbapenemase producers.

As per CDC Hospital Infection Control Practices Advisory Committee (HICPAC) in a hospital with CRE recommended guidelines which are as follows:

If there is a sporadic detection of CRE, it should be placed on contact precautions. The epidemiology and infection control staff should be alerted. The acute facilities should review records for at last 6-12 months for the occurrence of such cases. If found positive, then point prevalence survey should be carried out (i.e., one single round of active surveillance cultures) to identify patients colonized with CRE.

If CRE is routinely recovered including many cases admitted from the community where CRE is endemic, intensified infection control strategy should be adopted. This includes allocating additional resources for surveillance and control for aggressive infection control strategy and in US hospitals this has given dividend as CRE is low in these hospitals.

- Aggressive Infection Control strategy
- Contact precautions in managing patients CRE
- Implementing CLSI guidelines for detection of carbapenemase production.

3. MATERIALS AND METHODS

Bacterial Isolates

We conducted a cross-sectional study in the Dhaka Medical College Hospital, Dhaka, Bangladesh during January 2016 to July 2016. Total 120 laboratory samples from wound swab were collected. Fifty samples were from the patients of Burn unit and seventy were from other units (i.e. Surgery, Medicine and Gynecology & Obstetrics) of Dhaka Medical College Hospital.

Identification of species

Samples collected from different sources were inoculated in blood agar and MacConkey agar media and incubated at 37°C aerobically for 24 hours. Incubated plates were then examined for the presence of colonies of bacteria. All the organisms were identified by colony morphology, hemolytic criteria, staining character, pigment production and biochemical test as per standard technique (Cheesbrough, 1998). The isolated organisms were inoculated in TSI, MIU, citrate agar media and organisms were identified following chart of Colie *et al.* (1995). From the non-lactose fermenting colonies on MacConkey agar media, isolates were identified as *Pseudomonas aeruginosa* if they were oxidase positive, a triple sugar iron (TSI) agar reaction of alkaline over no change, motile, indole and urease negative in motility-indole-urea (MIU) agar media, citrate negative in Simmons citrate agar media and grew at both 37^0C and 42°C.

Antimicrobial susceptibility test

Susceptibilities to antimicrobial agents of all isolates were done by Kirby Bauer modified disk diffusion technique using Mueller Hinton agar plates and zones of inhibition were interpreted according to CLSI guidelines (CLSI, 2010). Antibiotic discs such as ceftriaxone (30 µg), ceftazidime (30 µg), cefepime (30 µg), amoxiclav

(amoxicillin 20 µg& clavulanic acid 10 µg), ciprofloxacin (5 µg), gentamycin (10 µg), amikacin (30 µg), imipenem (10 µg), meropenem (10 µg) and colistin (10 µg) were used to see the sensitivity patterns of the organisms.

Preparation of inoculums

Using a sterile wire loop, 3-5 well-isolated colonies of test organisms were emulsified in 3 ml of sterile normal saline. The turbidity of the suspension was compared with McFarland turbidity standard 0.5 by adding normal saline and placing a printed card behind the test and standard inoculums under proper light Both control and test inoculums were prepared.

Inoculation of test organisms

A sterile swab stick was placed on the bottom of the inoculum. The swab stick was pressed and rotated against the side of the tube above the level of the suspension for removal of extra fluid. The swab was streaked evenly over the surface of Mueller Hinton agar plate in three directions rotating the plate approximately 60°C. The inoculating plate was then allowed to dry for 3-5 minutes.

Antibiotic discs placement

Antibiotic discs were placed on the inoculating plate 15 mm away from the edge of the plate and 25 mm apart from one disc to another. Within 30 minutes of placement of antibiotic discs, the inoculated plates were incubated aerobically at 37°C overnight.

Interpretation of zone of diameter

After overnight incubation, the test plates were examined to ensure the confluence growth of the organism. The zone of inhibition was measured in mm using a ruler on the undersurface of the plate and evaluated with the standard chart of CLSI guideline (CLSI, 2010) (Appendix -IV). The examined clear zone of inhibition around the disc on the test organisms were interpreted as resistant, intermediate and sensitive.

Preservation of isolated organism

With all aseptic precautions with the help of a straight wire loop, few colonies of each organism were stabbed into nutrient agar base slants and incubated overnight at 37°C. Then the slants were immersed with sterile liquid paraffin (to exclude air and reduce dehydration) and were preserved in a refrigerator at 4°C. Subcultures were done every month into a fresh slant and were kept at 4°C.

The operational definition of ESBL producers

Organisms which are resistant to penicillin, first, second and third generation cephalosporin and aztreonam (but not cephamycins or carbapenems) and inhibited by beta-lactamase inhibitors such as clavulanic acid are considered as ESBL producers (CDC, 2011).

Screening for ESBL producers by double disc synergy assay (Collee *et al*., 1996)

This test was performed on Mueller Hinton agar. A disc containing ceftazidime 30 µg and a disc containing amoxicillin plus clavulanic acid (20 µg+10 µg) were placed 20-25 mm apart (center to center). The Mueller Hinton agar plate was incubated at 37°C for 24 hours. A clear extension of the edge of inhibition zone of cephalosporin disc towards amoxiclav disc was interpreted as ESBLs production.

Phenotypic detection of MBL producers

MBL producers were detected phenotypically among the isolated carbapenem-resistant *P. aeruginosa* by Hodge test and The Double Disk Synergy Test.

Hodge test

In this test, lawn culture was done on a Muller Hinton agar (MHA) plate with an overnight broth culture of *E. coli* ATCC 25922; the opacity was adjusted to 0.5 Mcfarland's standard. Then, imipenem resistant *P. aeruginosa* (test strain) was inoculated by lawn culture on the same plate. A 10µl of 50 mM zinc sulfate solution

was added on the imipenem disk and the plates were incubated at 37^0C overnight. The presence of a distorted zone of inhibition was interpreted as a positive result for carbapenem hydrolysis screening by the Hodge test.

The Double Disk Synergy Test

An overnight broth culture of the test strain (opacity adjusted to 0.5 Mcfarland opacity standards) was inoculated on a Muller Hinton agar plate. After drying, a 10 µg imipenem disk and a blank filter paper disk (6 mm in diameter, Whartman filter paper no.2) were placed 10 mm apart from edge to edge. 10 µl of 50 mM zinc sulfate solution was added to the 10 µg imipenem disk. Then, 10 µl of 0.5 EDTA (Sigma, USA) was applied to the blank filter paper disk. After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted as DDST positive.

Molecular detection of MBL producers

The standard for identification of carbapenemases is based on the use of molecular techniques, mostly PCR (Miriagou *et al.,* 2010; Nordman *et al.,* 2011). PCR performed on colonies may give results within 4-6 hours with excellent sensitivity and specificity.

Polymerase Chain reaction (PCR) for detection of specific β-lactamase gene

PCR is the most sensitive molecular method for detection of specific genes by amplifying the target DNA. DNA containing target sequence is denatured at 90-94^0C, cooled to a temperature for specific annealing of the two DNA primers and then the primers are extended by *Taq* polymerase at 72^0C across the target sequence of each strand. Thus the number of a target molecule is doubled and the first PCR cycle is completed. About 30 repetitions of these basic cycles amplify the target DNA 2^{30} times. After electrophoresis of amplified DNA, It is visualized by ultraviolet (UV)

illumination. It is a highly reliable method and is able to detect MBL producers accurately rather than any of the phenotypic methods (Bradford, 2001; Shibata *et al.,* 2003).

DNA Extraction

Bacterial DNA was extracted by the phenol-chloroform method. A phenol-chloroform extraction is a liquid-liquid extraction. A liquid-liquid extraction is a method that separates mixtures of molecules based on the differential solubility of the individual molecules in two different immiscible liquids (Stenesh, 1989).

The protocol used is presented below:

1. 1.5 ml of the overnight bacterial culture (grown in LB medium) was transferred to a 1.5 ml Eppendorf tube and was centrifuged at max speed for 1 min to pellet cells.
2. The supernatant was discarded.
3. The cell pellet was resuspended in 600 µl lysis buffer and was vortexed to completely resuspend cell pellet.
4. Incubation was done for 1 hour at 37 °C.
5. An equal volume of phenol/ chloroform/ isoamyl alcohol (25: 24: 1) was added and mixed well by inverting the tube until the phases are completely mixed.
6. Spinning was done at max speed for 5 min at room temperature. There was a white layer (protein layer) in the aqueous phenol/chloroform/isoamyl alcohol interface.
7. The upper aqueous phase was carefully transferred to a new tube.
8. To remove phenol, an equal volume of chloroform was added to the aqueous layer. Again, it was mixed well by inverting the tube.
9. Spinning was done at max speed for 5 min.
10. The aqueous layer was removed to a new tube.

11. To precipitate the DNA, 2.5 or 3 volume of cold ethanol (store ethanol at -20°C freezer) was added and mixed gently.

12. The tube was incubated at -20 °C for 30 min or more.

13. The tube was spun at max speed for 15 min at 4 °C.

14. The supernatant was discarded and the DNA pellet was washed with 1 ml 70% ethanol.

15. The tube was spun at max speed for 3 min. The supernatant was carefully discarded and the DNA pellet was air-dried.

16. DNA was resuspended in TE buffer.

17. Isolated DNA was stored at -20°C for further use.

Table 3.1: Sequence of selected primer

Gene	Sequence	Amplicon Size	Reference
NDM-1	Forward: ACCGCCTGGACCGATGACCA Reverse: GCCAAAGTTGGGCGCGGTTG	200 bp	Farzana *et al.,* 2013

Preparation of Master Mix

For PCR, reagents used along with the primers are Taq polymerase 5µl/ml (Thermofisher), 10x PCR buffer (Invitrogen, India), deoxynucleoside triphosphates (dNTPs) 2.5mM, $MgCl_2$ 50mM and nuclease-free water as required.

Table 3.2: PCR components for NDM-1 gene and their volume

PCR COMPONENTS	VOLUME
10X Reaction Buffer	5 µL
dNTPs	1 µL
Nuclease-free water	33.8 µL
Forward Primer (10 µM)	2.5 µL
Reverse Primer (10 µM)	2.5 µL
Taq Polymerase (3U/ml)	0.2 µL
Template DNA	5 µL
Total Volume	50 µL

Table 3.3: Thermal cycle condition for NDM-1

Steps	Temperature	Time
Initial Denaturation	95	10 min
30 cycles	95	1 min
Annealing	63	45 sec
Extension	72	1 min 30 sec
Final Extension	72	10 min

Gel Electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA, and proteins) and their fragments, based on their size and charge. The PCR products were further investigated through agarose gel electrophoresis. The PCR products were loaded into a gel with 6x loading dye. 100 bp ladder was used to estimate the size of the template DNA. The gel was run for 50 minutes at 80V. DNA was visualized using ethidium bromide under ultraviolet transilluminator.

4. RESULTS

A total of 120 laboratory samples collected from wound swab were included in this study. Fifty samples were from the patients of Burn unit and seventy were from other units (i.e. Surgery, Medicine and Gynecology & Obstetrics) of Dhaka Medical College Hospital.

Table 4.1 demonstrates the distribution of various species of gram-negative bacteria isolated from wound swab. Among the isolated gram-negative bacteria, 58 (48.33%) were *P. aeruginosa*, 27(22.5%) were *Klebsiella pneumoniae,* 20 (16.67%) were *Esch. coli*, 13 (10.83%) were *Proteus spp.* And 2 (1.67%) were Acinetobacter baumannii.

Table 4.1: Distribution of isolated gram negative bacteria

Species of bacteria	Number	%
Pseudomonas aeruginosa	58	48.33
Klebsiella pneumonia	27	22.5
Esch. Coli	20	16.67
Proteus spp.	13	10.83
Acinetobacter baumanni	2	1.67
Total	120	100.00

Among the 58 *P.aeruginosa,* 28 were isolated from the samples of the burn unit and 30 were isolated from the samples of other units.

Antimicrobial resistance pattern of isolated organisms are shown in Table 4.2. Regarding resistance pattern of *Pseudomonasn aeruginosa,* 89.65% were resistant to amoxiclav, 82.75% to amikacin, 87.93% to ciprofloxacin, 84.48% to ceftazidime,

77.58% to cefepime, 91.37% to ceftriaxone, 79.31% to gentamicin, 31.03% to imepenem, 36.20% to meropenem and 5.17% to colistin.

Table 4.2: Antimicrobial drug resistance pattern among different species of bacteria

Antimicrobial drugs	*Pseudomonas aeruginosa* (n=58) N (%)	*Klebsiella Pneumoniae* (n=27) N (%)	*E.coli* (n=20) N (%)	*Proteus spp.* (n=13) N (%)	*Acinetobacter baumannii* (n=2) N (%)
Amoxiclav	52 (89.65)	25 (92.59)	19 (95.00)	13 (1000	2 (100)
Amikacin	48 (82.75)	23 (85.18)	16 (80.00)	11 (84.61)	2 (100)
Ciprofloxacin	51 (87.93)	24 (88.88)	18 (90.00)	12 (92.30)	2 (100)
Ceftazidime	49 (84.48)	20 (74.07)	17 (85.00)	11 (84.61)	2 (100)
Cefepime	45 (77.58)	21 (77.77)	17 (85.00)	12 (92.30)	2 (100)
Ceftriaxone	53 (91.37)	25 (92.59)	18 (90.00)	11 (84.61)	2 (100)
Gentamicin	46 (79.31)	21 (77.77)	15 (75.00)	10 (76.92)	2 (100)
Imipenem	18 (31.03)	9 (33.33)	5 (25.00)	3 (23.07)	1 (50)
Meropenem	21 (36.20)	11 (40.74)	6 (30.00)	4 (30.76)	2 (100)
Colistin	3 (5.17)	1 (3.7)	0 (0.00)	0 (0.00)	0 (0.00)

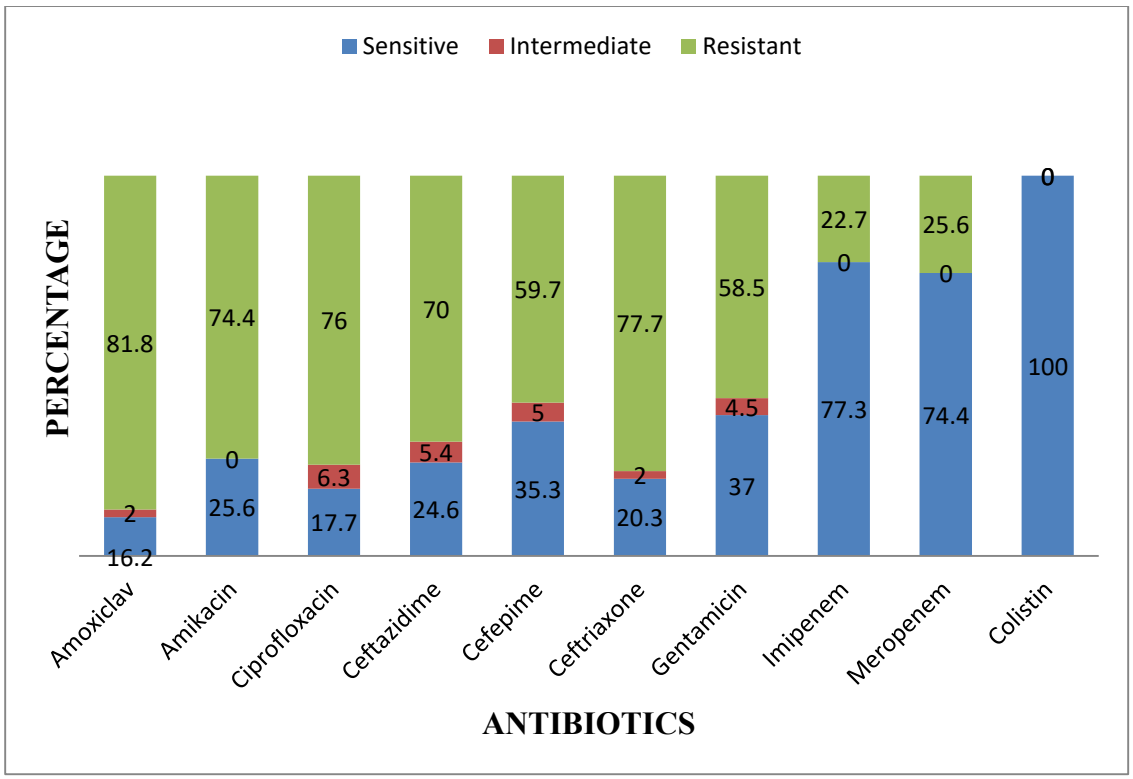

Figure 4.1: Antimicrobial susceptibility pattern of *P. aeruginosa* isolated from the sample of medicine, surgery, gyne & obstetrics units

Figure 4.1 shows the antimicrobial susceptibility pattern of *P. aeruginosa* isolated from the sample of surgery, medicine, gyne & obstetrics unit. Among the 30 isolated *P. aeruginosa,* 87.8% were resistant to amoxiclav, 74.4% to amikacin, 76% to ciprofloxacin, 70% to ceftazidime, 59.7% to cefepime, 77.7% to ceftriaxone, 58.5% to Gentamicin 22.7% to imipenem, 25.6% to meropenem and no bacteria were resistant to colistin.

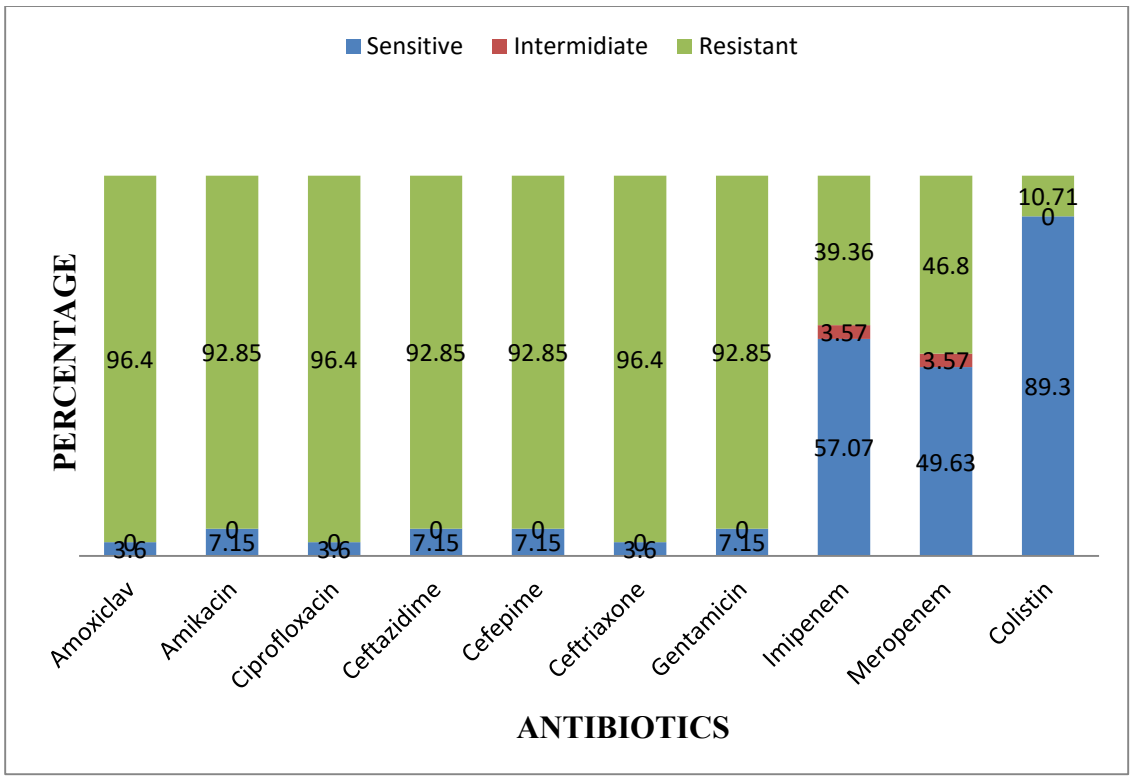

Figure 4.2: Antimicrobial susceptibility pattern of *P. aeruginosa* isolated from the sample of the burn unit

Figure 4.2 shows the antimicrobial susceptibility pattern of *P. aeruginosa* isolated from the burn unit. Among the 28 samples, 96.4% were resistant to amoxiclav, ciprofloxacin, and ceftriaxone. 92.85% were resistant to amikacin, gentamicin, ceftazidime, and cefepime. Among the samples of burn unit 39.36% were resistant to imipenem, 46.8% were resistant to meropenem and 10.71% were resistant to colistin.

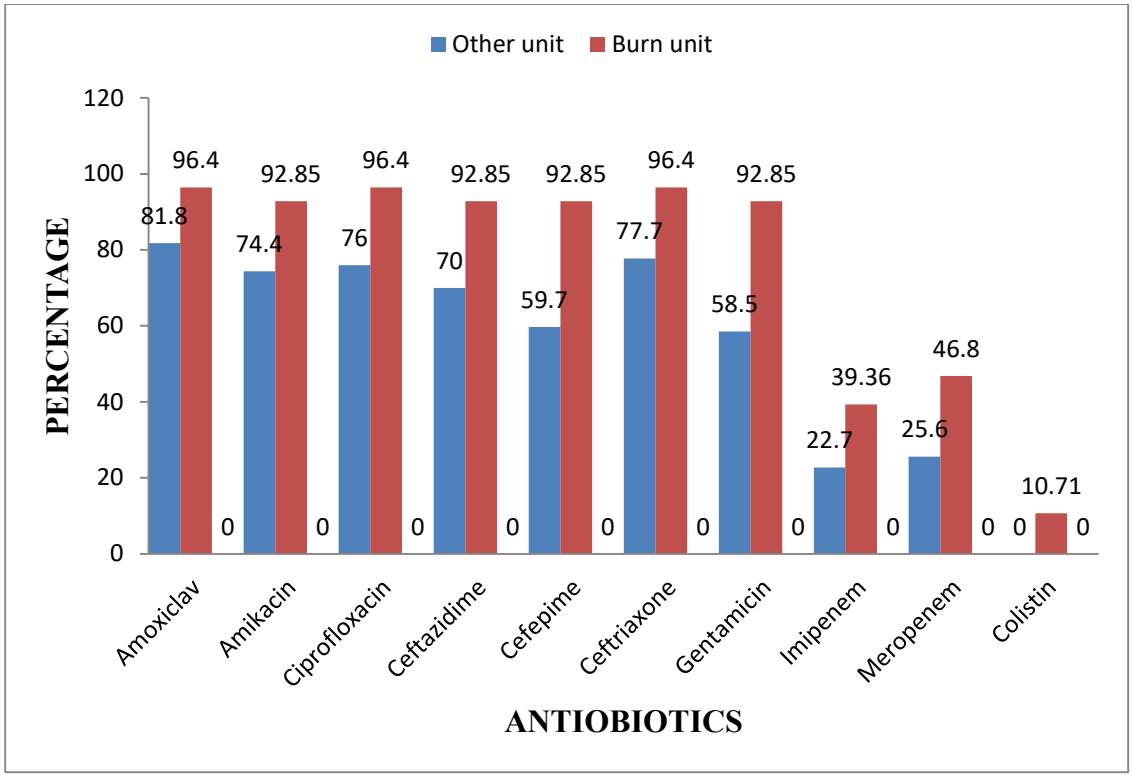

Figure 4.3: Comparison of antibiotic resistance pattern of *P.aeruginosa* isolates collected from the burn and other units

Figure 4.3 shows the comparison of antibiotic resistance pattern of *P. aeruginosa* isolates collected from samples of burn and other units. Samples from burn unit show more resistant to each and every antibiotic.

Table 4.3: Distribution of ESBL producing bacteria identified by DDS test

Species	Total number	ESBL producers	%
P. aeruginosa	58	17	29.31%
K. pneumonia	27	9	33.33%
E. coli	20	7	35%
Proteus spp.	13	4	30.76%
Acinetobacter baumannii	2	1	50%
Total	120	38	31.67%

Table 4.3 shows the distribution of ESBL producers among different species of gram-negative bacteria. Among 120 isolated gram-negative bacteria, 38 (31.67%) ESBL producers were detected by DDS test. Total 17 (29.31%) of 58 *P. aeruginosa* were ESBL producers. In the case of *K. pneumonia* and *E. coli,* 33.33% and 35% were ESBL producers respectively.

Table 4.4 shows the distribution of MBL producers among different species of gram-negative bacteria. Among 120 isolated gram-negative bacteria, 40 (33.33%) MBL producers were detected by DDS test. Total 20 (34.48%) of 58 *P. aeruginosa* were MBL producers. In the case of *K. pneumonia* and *E. coli,* 37.03% and 30% were ESBL producers respectively.

Table 4.4: Distribution of MBL producing bacteria identified by DDS test

Species	Total number	MBL producers	%
P. aeruginosa	58	20	34.48%
K. pneumonia	27	10	37.03%
E. coli	20	6	30%
Proteus spp.	13	3	23.07%
Acinetobacter baumannii	2	1	50%
Total	120	40	33.33%

Total 24 carbapenem-resistant (3 to only imipenem, 6 to only meropenem and 15 to both) *Pseudomonas aeruginosa* were detected. Among the carbapenem-resistant, *Pseudomonas aeruginosa* 17 (70.83%) were detected as MBL producers by Hodge test and 20 (83.33%) were detected as MBL producers by double disk synergy (DDS) test. All 24 carbapenem-resistant *Pseudomonas aeruginosa* were investigated for the presence of *the bla*NDM-1 gene through PCR. Out of 24 carbapenem-resistant, *Pseudomonas aeruginosa* 6 (25%) were NDM-1 positive by PCR. Five of them were from samples of burn unit and one was from the sample of other units.

Table 4.5: Distribution of NDM-1 gene among carbapenem-resistant *P. aeruginosa*

Collection unit	Carbapenem-resistant *Pseudomonas aeruginosa*	NDM-1 Producer	%
Burn unit	15	5	33.33%
Other units	9	1	11.11%
Total	24	6	25%

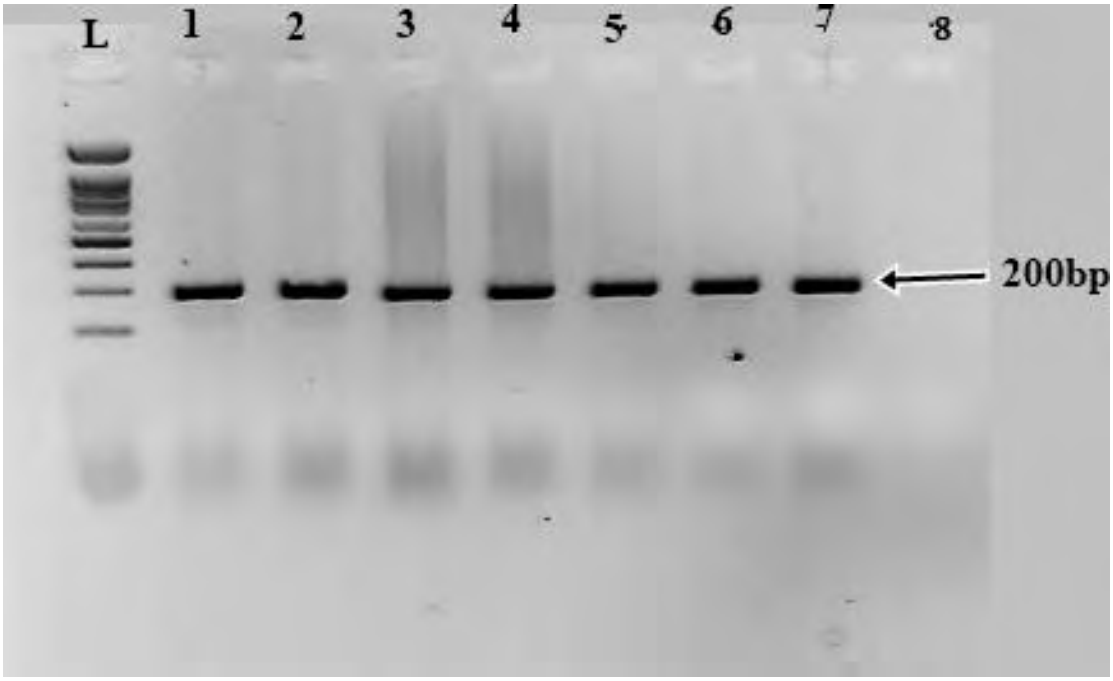

Figure 4.4: Gel electrophoresis of a 100 bp ladder and PCR product of *bla*NDM-1

Figure 4.4 shows the gel electrophoresis result of *bla*NDM-1 gene. Lane one shows the 100 bp DNA ladder, in lane 1 there is a positive control *Pseudomonas aeruginosa* ATCC 27853 and in lane 8 there a negative control *E.coli* ATCC 25922. The 6 positive samples (lane2-7) showed a band at 200 bp which confirmed the presence of the *bla*NDM-1 gene.

Table 4.6: Antibiotic susceptibility pattern of NDM-1 producing *Pseudomonas aeruginosa*

Antimicrobial drugs	Sensitive N (%)	Resistant
Amoxiclav	0 (0%)	6 (100%)
Amikacin	0 (0%)	6 (100%)
Ciprofloxacin	0 (0%)	6 (100%)
Ceftazidime	0 (0%)	6 (100%)
Cefepime	0 (0%)	6 (100%)
Ceftriaxone	0 (0%)	6 (100%)
Gentamicin	0 (0%)	6 (100%)
Imipenem	0 (0%)	6 (100%)
Meropenem	0 (0%)	6 (100%)
Colistin	5 (83.33%)	1 (16.67%

Table 4.6 shows the antibiotic susceptibility pattern of NDM-1 producing *Pseudomonas aeruginosa.* NDM-1 producing *P. aeruginosa* were resistant to all antibiotics except colistin. Only one is resistant to colistin.

5. DISCUSSION

The emergence of antibiotic resistance is a matter of great concern, particularly in hospitals. Antibiotic-resistant bacteria appear to be biologically fit and capable of causing serious life-threatening infections. The increase in antibiotic resistance among gram-negative bacilli, such as Enterobacteriaceae group, *Pseudomonas aeruginosa,* and others, is a notable example and how bacteria can procure, maintain and express new genetic information that can confer resistance to one or several antibiotics. Resistance in gram-negative bacteria is a serious problem and calls for effective infection control measures to curb their dissemination (Walsh *et al.,* 2005; Bradford, 2001).

Strains of *P. aeruginosa* cause disease in hospitalized patients, predominantly pneumonia, urinary tract infection, as well as, skin and soft tissue infection (Cristino, 1999). The increased involvement of this ubiquitous organism in infections is due to a number of factors, including the growing numbers of invasive procedures and immunocompromised patients together with the increased use of antibiotics, which has promoted the selection of resistant organism. Patients in the intensive care unit, oncology departments, bum units, and surgery wards frequently show multidrug-resistant isolates, which contributes to high morbidity and mortality (Giamarellos *et al.,* 2006).

In this study, 120 gram-negative bacteria were isolated from wound swab which is collected from the burn and other units of DMCH. A study carried out by Saha *et al.* (2011) at DMCH on burn patients found *P. aeruginosa* as the highest isolated organism (39%). In this study, *P. aeruginosa* was again found to be commonly encountered (48.33%) pathogen. *Klebsiella pneumonia* (22.50%) was the second most commonly isolated organism (Table 4.1).

In this study, *P. aeruginosa* was 31.03% resistant to imipenem and 36.20% resistant to meropenem (Table 4.2). The findings are not similar to the previous study by Saha *et al.* (2011) who found that 98.72% *P. aeruginosa* were sensitive to imipenem. This increasing trend of resistance may be due to selective pressure on carbapenems as a result of increased use and an increase in carbapenemase production along with intrinsic resistance by *P. aeruginosa.* A study in a tertiary-care hospital in India reported similar rates (28.5%) of imipenem resistance among *P. aeruginosa* (Kaur *et al.,* 2017). In Japan, the rate of resistance to carbapenem increased from 19.3% in 1998 to 38% in 2002 (Fritsehe *et al.,* 2005). A study in a tertiary-care teaching hospital in southern Brazil reported higher rates (56.7-58.3%) of imipenem resistance among *P. aeruginosa* (Yan *et al.,* 2001). Zavascki *et al. (*2005) identified carbapenem exposure as a risk factor for acquisition of imipenem resistant *P. aeruginosa* in a case-control study. Rasmussen and Bush (1997) predicted that an increase of MBL-producing organism was inevitable, given the more frequent use of carbapenems.

The result of this study indicates that *P. aeruginosa* isolated from samples of burn unit is more resistant to carbapenem antibiotics than other units (Figure 4.3). This result is similar to the result of the study carried out by Jobayer *et al. (*2017) in DMCH who also found increased resistance among bacteria isolated from burn patients. This increasing trend of resistance again may be due to selective pressure as a result of increased use of carbapenem antibiotics in the burn unit.

The present study observed 29.31% *P. aeruginosa* as ESBL producer by double disc synergy test (Table 4.3). In contrast to the present study, Farzana *et al.* (2013) reported 12.82% *Pseudomonas spp.* as ESBL producers by double disc synergy test. This increased proportion of ESBL producing *Pseudomonas* in the present study might be due to the fact that ESBL producers have increased in recent years among gram-

negative isolates as a result of increased use of antibiotics creating selective pressure (Paterson, 2006; Bouijilat *et al.,* 2011). In India, Kaur *et al.* (2017) reported 25% ESBL producers among gram-negative bacteria. In this study, 31.67% gram-negative isolates are ESBL producers identified by DDS test.

In this study, 33.33% gram-negative isolates are MBL producers identified by the double disk synergy test. In a tertiary care hospital in India 25.3% gram-negative bacteria and 34.6% *Pseudomonas spp.* are reported as MBL producers (Kaur *et al.,* 2017). In this study, 24 carbapenem-resistant *P. aeruginosa* were searched for MBL production (Hodge test and DDS test) since earlier reports suggested that MBLs had carbapenemase activity (Bebrone, 2007). The result showed that 34.48% *P. aeruginosa* were MBL producer. Risk factors for acquiring an carbapenem- resistance included previous carbapenem use, longer duration of hospital admission, ICU admission, urgent surgery, being on total parenteral nutrition and using tubes and catheter such as central venous catheter, endotracheal tube, urinary catheter and nasogastric tube (Meyer *et al.,* 2003; Cisneros *et al.,* 2005). The previous study in Bangladesh revealed 43% MBL producers among the imipenem resistant *P. aeruginosa* (Nasrin *et al.,* 2010).

The present study observed 6 (25%) NDM-1 positive *P. aeruginosa* among 24 carbapenem-resistant *P. aeruginosa* (Table 4.5). A study by Kumarasamy *et al.,* (2010) reported NDM-1 producing bacteria from India, Pakistan, and the UK. NDM-1 producers were also detected in Europe (Struelens *et al.,* 2010), Australia (Poirel *et al.,* 2010) and America (Mulvey *et al.,* 2011). Interestingly, most of the cases from Europe, America, and Australia had a history of recent travel or hospital admission in the Indian subcontinent. Rolain *et al.* (2010) suggested that the acquired carbapenemases have been mainly restricted to certain geographical areas and to

specific bacterial species, and outbreaks as well as spread in other countries have been often associated with imported cases from countries where the bacteria have been endemic. Population mobility is known to be the main factor in globalization and in some way spreading of antimicrobial drug-resistant organisms. Organisms containing *bla*NDM-1 are now alarmingly rising worldwide and pose therapeutic failure (Kumarasamy *et al.,* 2010; Struelens *et al.,* 2010).

A previous study in Bangladesh demonstrated 3.5% NDM-1 producers among the imipenem resistant organisms and no imipenem resistant *Pseudomonas* harbored NDM-1 (Islam *et al.,* 2012). The present study found a higher proportion (25%) of NDM-1 producers among *P. aeruginosa* in DMCH. The increasing percentage of this new resistance mechanism might be due to healthcare associated acquisition of *bla*NDM-1 in hospitalized patients of Bangladesh which has also found in different parts of the world (Struelens *et al.,* 2010). Inappropriate and non-prescription antibiotics use might be the probable cause of the development of this new resistance mechanism in this subcontinent (Mamun *et al*., 2006). In another study, Farzana *et al*. (2013) reported 22.86% NDM-1 producers among total 35 imipenem resistant gram-negative bacteria at DMCH. The ability of NDM-1 to spread not only among *Enterobacteriaceae* but also among other bacterial families, like *Pseudomonaceae*, implies the possibility for numerous new NDM-1 cases to be detected in the near future. Looking at the chronology of VIM-1 spreading, a rapid emergence of new NDM-1 cases might be expected (Toleman *et al.,* 2004).

In India, NDM-1 producing organisms were mostly acquired from the community (Kumarasamy *et al.,* 2010). It reflects that though most NDM-1 producers were isolated from hospitalized patients, originally this resistance mechanism may have extended from the community. Timothy Walsh and colleagues identified *bla*NDM-1

carrying bacterial species from *Enterobacteriaceae, Aeromonadaceae, Vibrionaceae* and *Pseudomonadaceae*, and some other non-fermenters, in New Delhi water samples (Walsh *et al.,* 2011). It is possible that these organisms acquired *bla*NDM-1 from other gram-negative organisms in the environment through horizontal gene transmission. In a recent study, it is reported that 71% wastewater samples from hospital-adjacent areas were positive for NDM-1-producing bacteria, as evidenced by phenotypic tests and the presence of the *bla*NDM-1 gene (Islam *et al.,* 2017). Hasan *et al.* (2010) reported that in a study carried in Bangladesh on 150 faecal samples from gulls along the coastline and 238 fecal samples from crows in the grounds of Rajshahi Medical College Hospital and Chittagong Medical College Hospital to detect *bla*NDM-1 expression with a belief that birds that live close to people might be transmitting ESBL and MBL producing bacteria from hospital to community. No NDM-l was detected in Bangladeshi bird feces but environmental reservoirs of NDM-1 carrying bacterial strains need to be identified.

In this study, all NDM-1 producing *P. aeruginosa* were resistant to imipenem, meropenem, ceftriaxone, ceftazidime, amoxiclav, amikacin, gentamicin, and ciprofloxacin (Table4.6). A study carried out by Poirel *et al*. (2010) also reported a multidrug-resistant *Esch. coli* that harbored NDM-1 gene and was resistant to all β-lactams (including carbapenems), all aminoglycosides, fluoroquinolones, nitrofurantoin, and sulfonamides, remaining susceptible only to tetracycline, fosfomycin, and colistin. This may be due to the fact that Metallo-β-lactamases have significant carbapenemase activity and resistant to β-lactamase inhibitor such as the clavulanic acid (Cornaglia *et al.,* 2011; Walsh *et al.,* 2005). Moreover, plasmid carrying the *bla*NDM-1 gene are diverse and can harbor a high number of resistance genes associated with other carbapenemase genes (OXA-48 types, VIM types),

plasmid-mediated cephalosporinase genes, ESBL genes, aminoglycoside resistance genes (16S RNA methylases), macrolide resistance genes (esterase) and sulfamethoxazole resistance genes as source of multidrug resistance and pan-drug resistance (Kumarasamy *et al.,* 2010; Nordmann *et al.,* 2011). A study carried out in Bangladesh by Islam et al. (2012) also reported co-harboring of *the bla*CTX-M-1 group, *bla*TEM, *bla*SHV, *bla*OXA-1 group, *bla*VIM, and *bla*CMY genes. Co-occurrence of 16S rRNA methylase genes (*arm*A, *rmt*B, *rmt*C), plasmid-borne quinolone resistance genes (*qnr*) were also reported.

The global dissemination of drug resistance mechanism posses now limited therapeutic options for the management of serious infections. The finding of the current study also reflects this emerging situation in Bangladesh. With respect to antimicrobial susceptibility, imipenem and meropenem as a single agent were the most sensitive antibiotic for the treatment of infection against *P. aeruginosa* isolated from wound swab. But for the MBL producers, colistin was most active drug though colistin resistance is emerging. Moreover, the clinical utility of colistin was limited because of neurotoxicity and nephrotoxicity (Kock-Weser *et al., 1970*). The combination of two or more antimicrobial agents may provide a better effect. The outcome of combinations of more than two drugs can also be evaluated. It has become increasingly clear that over-the-counter sales and the indiscriminate use of antibiotics in low- and middle-income developing countries play an important role in the spread of antimicrobial resistance genes (Okeke *et al.,* 1999). Considering the grave scenario of antibiotic resistance in our country, it is high time that all clinical laboratories start detecting the ESBLs and MBLs routinely and accurately. This study also helps us to understand the need for more precaution in the use of antibiotics.

6. CONCLUSION

Pseudomonas aeruginosa is an important pathogen in wound infection. Among the isolated organisms 48.33% were *P. aeruginosa* and 41.37% of them were carbapenem-resistant. Among the carbapenem-resistant isolates, NDM-1 is detected in 25% of samples. Samples from burn unit showed more resistance and were more NDM-1 producers than other units. High antimicrobial resistance was observed against all β-lactam and non-β-lactam antibiotics by the NDM-1 producers. Colistin as a single agent was most effective against NDM-1 producers though the present study observed the emergence of colistin resistance. NDM-1 has been detected in a significant percentage of Gram-negative bacteria in Bangladesh. The potential spread of *bla*NDM-1 among bacterial populations is a reason for concern and the observations from this study further highlight the emerging therapeutic challenge in Bangladesh. Early detection of this resistance mechanism, implementation of strict antimicrobial policies and infection control programs may avoid the rapid dissemination of these organisms.

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8. APPENDICES

APPENDIX-I

Preparation of Media

Blood agar

Dehydrated blood agar base: 40 grams.

Distilled water: 1000 ml.

P^H: 6.8

Media was dissolved in distilled water. P^H adjusted at 7.2. Autoclaved at 121°C for 15 minutes under 15 Lbs pressure, cooled down at 50°C, 7% sheep blood (defibrinated, aseptically collected) was added, mixed well and poured into sterile Petri dishes.

McConkey agar

Dehydrated MacConkey agar: 50 grams.

Distilled water: 1000 ml.

Media was dissolved in distilled water, P^H adjusted at 7.2 and autoclaved at 121°C on 15 Lbs pressure for 15 minutes. After cooling at about 55°C, poured into sterile Petri dishes.

Muller-Hinton agar

Dehydrated MH base: 38 grams.

Distilled water: 1000 ml.

PH: 4± .02

Autoclaved at 121°C on 15 Lbs pressure for 15 minutes, cooled down at 55°C, poured into sterile Petri dishes.

Kligler iron agar (KIA)

Dehydrated KIA media: 65 grams.

Distilled water: 1000 ml.

The ingredient was dissolved by boiling and P^H was checked, distributed into a screw-capped test tube in 5 ml amounts. Autoclaved at 121°C on 15 lbs pressure for 15 minutes and kept it to become solidified in such a position that gave a slant and a deep butt.

Simmons citrate agar

Dehydrated media: 24.2 grams.

Distilled water: 1000 ml.

P^H: 6.8

The ingredients were dissolved by boiling and P^H was checked, distributed into screw-capped test tubes. Autoclaved at 121°C on 15 lbs pressure for 15 minutes and allowed to solidify as slopes.

Motility-indole-urea (MIU) agar

Dehydrated media: 18 grams.

Distilled water: 950 ml.

$P^{H;}$ 6.8 ±.02

The media was boiled to dissolve completely, autoclaved at 121°C on 15 lbs pressure for 15 minutes and cooled down at 55^0C. Then added 5 ml of sterile 4% urea solution (FD048) per 95 ml and dispensed into a test tube.

Trypticase soy broth

Dehydrated TSB base: 30 grams

Sodium polyanethol sulphonate: 0.5 grams

Distilled water: 1000ml

P^H: 7.3

PABA: 0.05 grams

Autoclaved at 121^0C for 15 minutes under 15 lbs pressure and poured into falcon tubes or Eppendorf tubes according to necessity.

Nutrient agar slant

Dehydrated blood agar base: 30 grams

Distilled water: 1000ml

P^H: 7.4

The ingredients were distributed into small size screw-capped vials and Autoclaved at 121^0C for 15 minutes under 15 lbs pressure. The vials were kept into slanting position till media were solidified.

APPENDIX-II

BUFFERS AND REAGENTS

Name of the Buffer or reagents	Component	Amount (g/L)
1 M Tris-HCL	Tris	121.14
	HCL	As required to adjust the P^H
	Final P^H	8.00
0.5 M EDTA	EDTA	186.00
	NAOH	As required to adjust the P^H
	P^H	8.00
1X TE Buffer	1 M Tris-HCL	10.00
	0.5 M EDTA	2.00
	P^H	8.00
1X TBE Buffer	Tris base	10.80
	Boric acid	5.50
	0.5 M EDTA	4.00
	P^H	8.00
Lysis Buffer	1X TE Buffer	9.34mL/10mL
	10% SDS	600 µL
	Proteinase K	60 µL

APPENDIX-III

LIST OF EQUIPMENT

INSTRUMENT	MANUFACTURER
Weighing Machine	Adam Equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator	Model: WIS-20R Daihan Scientific Companies, Korea
UV Transilluminator	Model: MD-20 Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer	Model: JSHS-180 JSR, Korea
Vortex Machine	VWR International
Microwave Oven	Model: MH6548SR LG, China
P^H Meter	P^H ep Tester Hanna Instruments, Romania
Eppendorf	Germany, Ireland
Refrigerator	(40C) Model: 0636 Samsung

APPENDIX-IV

Interpretative zone diameter (in mm) (CLSI, 2010)

Antimicrobial drug	**Resistant**	**Intermediate sensitive**	**Sensitive**
Imipenem (l0µg)	13	14-15	16
Meropenem (l0µg)	13	14-15	16
Ceftriaxone (30µg)	13	14-20	16
Ceftazidime (30µg)	14	15-17	18
Cefepime (30µg)	14	15-17	18
Amoxiclav (20/10µg)	13	14-17	18
Ciprofloxacillin (5µg)	15	16-20	21
Gentamicin (10µg)	12	13-15	16
Amikacin (30µg)	14	15-16	17
Colistin (l0µg)	10		11